PROTEASE INHIBITORS AND REPRODUCTION OF RENIFORM NEMATODE IN PINEAPPLE

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Abstract

Protease inhibitors (PI) are thought to serve as defense compounds against pathogen attack in many plant systems. PIs have been found in pineapple roots, and populations of *Rotylenchulus reniformis* remain untypically low for 6-9 months after pineapple planting. A potted plant experiment was conducted to determine if PIs present in pineapple roots affected nematode reproduction and could account for the observed field population dynamics. Pineapple plants with and without *R. reniformis* were harvested monthly. Roots were removed from the plant, weighed, shaken in NaOCl to determine number of nematode eggs, then cut into 1 cm pieces. Roots were homogenized in EDTA/phosphate buffer at pH 6.0 to extract PIs. An aliquot of the root extract was added to purified papain and colorimetric protein substrate, and inhibition activity determined. Pineapple PI activity increased for the first 6 months after planting (in both inoculated and not inoculated treatments), and was higher in nematode inoculated plants. Nematode reproduction (egg numbers) was not correlated to PI activity. A second potted plant experiment was conducted to determine if PI levels in pineapple roots varied along root length in the presence and absence of nematodes. PI activity increased in the presence of nematodes and this increase was concentrated in the basal portion of the roots where nematode densities were highest. These results support a defensive role for PI in pineapple roots. More work is necessary to elucidate the role of pineapple PIs in nematode population regulation.

Chapter 1

Literature Review

Introduction

Pineapple is a fruit of ancient importance in the Americas and has become a high-value crop worldwide since its dissemination from the New World was begun by the Spanish in the fourteenth century (Nakasone and Paull 1998). Global production in 2000 is estimated to be greater than thirteen million metric tons (FAO, 2001). The total value of fresh market and processed pineapple in Hawaii reported for 1999 was over $144 million (HASS 2001).

Reniform nematodes are widespread in the tropics, infecting a wide range of host plants, and are a major pest of pineapple (Rorbach and Apt 1986; Whitehead 1998). Mature females are sedentary, semi-endoparasites of roots. Damage to pineapples can be caused by more than 1 nematode/gram soil (Sipes et al., 2000; Whitehead 1998). Reniform nematodes feed on the cortical tissue of lateral roots and thus inhibit secondary root formation resulting in poorly developed root systems (Rorbach and Apt, 1986). Predictably, foliar symptoms of pineapple root infection by reniform nematodes are similar to nutrient or moisture stress symptoms (Caswell et al., 1990).

Pineapple

Pineapple, *Ananas comosus*, is a monocotyledonous perennial herb grown for its edible fruits and leaf fiber. Pineapple originated in the interior of

Venezuela and was brought to Hawai`i in the late 18th century (Nakasone and Paull, 1998). The plants are very heterozygous so many clone lines are derived from somatic mutations (Purseglove, 1988). 'Smooth Cayenne' is currently the most widely grown cultivar (Nakasone and Paull, 1998).

The plant is especially tolerant to drought because of special water storage cells and the Crassulacean Acid Metabolism (CAM) photosynthetic pathway, where carbon dioxide is converted into acids at night, which during the day are used to synthesize carbohydrates (Wee and Thongtham, 1991). This allows the stomata to be closed during the day, limiting water use. Even though the plant is very drought tolerant, the root system of pineapples is shallow and limited, so growth is slowed under dry conditions. Optimum rainfall is 1000-1500 mm per year (Purseglove, 1988). In dry places, irrigation helps to maintain growth and advance fruiting (Wee and Thongtham, 1991). The crop can grow on a wide range of soils but does not tolerate waterlogging.

When plants are 10-11 months old, flowering is generally induced with ethephon or α-naphthalene acetic acid (NAA) and fruits are harvested 7-8 months later (Wee and Thongtham, 1991). Chemical flower induction is easiest under relatively cool, short-day conditions (Nakasone and Paull, 1997). Pineapple is a perennial, so 1-2 secondary (ratoon) harvests may be made from suckers of the mother plant (Pursglove, 1988).

Reniform Nematode and Pineapple

Many diseases and pathogens attack the roots of pineapple. There are 3 nematode pests of major importance. *Pratylenchus brachyurus* is of limited significance in Hawai`i, while *Meloidogyne javanica* is a serious problem and *Rotylenchulus reniformis* is the most significant nematode pathogen of pineapple in Hawai`i. *Rotylenchulus reniformis*, or Reniform nematode, is widespread in the tropics, infecting a wide range of host plants (115 known plant species) (Whitehead, 1998). Reniform nematodes are significant in Hawai'i because they are evenly distributed large hectarages (Rohrbach and Apt, 1986).

Mature reniform females are sedentary, semi-endoparasites of roots. The nematodes feed on cortical parenchyma, pericycle or even phloem tissue. Reproduction is sexual but parthenogenesis is known to occur. Up to 70 eggs can be laid by a female in a gelatinous matrix. One life cycle is completed in about 30 days, depending on host and soil conditions (Whitehead, 1998).

The reniform nematode population on pineapples in Hawaii doesn't increase until 6-8 months after planting (Sipes and Schmitt, 1994). This long lag phase has been found with other nematodes on pineapple in other places, e.g., *M. javanica* in Australia (Stirling and Nikulin, 1993), and *Pratylenchulus* in Africa (Caswell et al, 1990).

Reniform nematode was first discovered in 1935 in Hawai`i. They became a problem in Hawaii because of agricultural practices starting in the 1940s. Pineapple was grown as a monoculture crop with a single cultivar that was a

good host for the nematode. The soil's pH dropped to a nematode friendly level through the use of ammonium sulfate fertilizer. Soil fumigation reduced the population of natural antagonists, so that those nematodes that survived increased to even higher levels (Rohrbach and Apt, 1986). The practice of long crop cycles followed by a long fallow period was abandoned as pineapples became more popular and production increased. Finally, soil moisture was not maintained so that plants were under water stress during part of their growth cycle and reniform flourishes under low water conditions, making it easier to attack the already stressed plant (Rohrbach and Apt, 1986).

Damage to pineapples can be caused by more than 1 nematode/gram soil (Whitehead 1998). Symptoms of infection of pineapple by reniform nematodes have been reviewed by Caswell et al (1990). Foliar symptoms exhibited by reniform nematode inoculated plants are similar to nutrient or moisture stress symptoms. Though the nematode feeds on the cortical tissue of the lateral pineapple roots, it doesn't seem to seriously affect elongation of the primary root, allowing the plant to retain good soil anchorage (Rohrbach and Apt, 1986). However, secondary root formation is inhibited resulting in poorly formed root systems. Since the roots do not regenerate, mother plant root health is essential for continued growth; if the root system is seriously damaged, subsequent ratoon crops are devastated, resulting in significant yield loss (Rorbach and Apt, 1986).

Several different methods are used to control reniform nematodes in pineapple. The emphasis is on protecting the young growing root system by reducing nematodes in the soil before planting. The primary means of achieving

this is through fumigation with the nematicide 1,3-dichloropropene (Rohrbach and Apt, 1986). If fields are not treated for nematodes, yield (marketable tons/ha) of both the plant crop and the 1st ratoon crop is lower than when treated with nematicides (1,3-D and fenamiphos) (Sipes and Schmitt, 1994). Fallow periods are very important for helping to reduce nematode numbers before fumigation, and planting non-host cover crops as a crop rotation may provide increasing control (Caswell et al., 1990). Post-plant non-fumigant nematicides such as fenamiphos are also sometimes necessary when multiple ratoon crops are desired. Pineapple clones that show resistance to reniform nematodes have undesirable agronomic characteristics and are excellent hosts for *P. brachyurus* (Py et al., 1984).

Protease Inhibitors (PI)

Plants have or make (in response to an attack) an array of defensive chemicals such as antibiotics, alkaloids, terpenes and proteins (such as enzymes, lectins, and enzyme inhibitors). Proteases are enzymes that digest protein. They may be either endopeptidases or exopeptidases. Endopeptidases are also called proteinases. Protease inhibitor (PI) proteins are among the defensive chemicals in plant tissues that are both developmentally regulated and induced in response to insect/pathogen attacks (Ryan, 1990). PI proteins form complexes with proteases and inhibit their proteolytic activity (Neurath, 1984). There are 8-10 PI families, grouped into four classes: serine proteases, cysteine

proteases, aspartic proteases, and metallo-proteases (Ryan, 1990). The first three classes are important in plants (Ryan, 1990).

Plant proteins are the foods of insects/pathogens that attack the plant. Animal pests and parasites have a variety of proteinases that assist them in invading host tissues, parasitizing nutrition and suppressing host immune responses (Ryan, 1990). Nematodes, for example, insert their stylet into a plant cell and the nematode is believed to release digestive enzymes to digest the contents, which are then extracted through the stylet (Whitehead, 1998). Other roles for PI in plant cells have been proposed, including the regulation of proteases in plant tissues such as seeds and storage roots to promote protein accumulation (Koiwa et al., 1997). However, most work has focused on their role in plant defense.

Orozco-Cardenas et al. (2001), have reviewed PI gene expression in plants as a result of pest attack, and proposed the following model: 1) a signal (systemin) is produced in response to wounding; 2) systemin induces linoleic acid production by cell membranes; 3) the linoleic acid is converted to jasmonic acid which then induces expression of genes coding for a secondary messenger (H_2O_2). H_2O_2 in turn activates PI genes. Jasmonic acid produced in the vascular bundle cells is thought to be translocated to other parts of the plant where H_2O_2, then PI are produced primarily in mesophyll cells and stored in the vacuole (Orozco-Cardenas et al., 2001). Systemic induction of PI may take place via signal transduction through both the xylem and phloem (Koiwa et al., 1997). Signal gene expression occurs rapidly after wounding and PI production has

been reported 4-24 hours after wounding or exposure to jasmonic acid (Bolter, 1993; Orozco-Cardenas et al., 2001).

Of the protease families, cysteine proteases (belonging to the papain super-family) are thought to be most important for proteolysis in the midgut of nematodes (Koiwa et al., 1997), and management of PI levels in plant roots may represent a new method of nematode control in crops. Recent work has demonstrated this potential. For example, expression of the cysteine protease inhibitor oryzacystatin-I (Oc-I) in hairy roots of tomato reduced the growth and development of potato cyst nematode, *Globodera pallida* (Urwin et al., 1995). Other workers observed that cowpea trypsin inhibitor (CpTI) expressed as a transgene in tobacco leaves reduced feeding on the leaves by tobacco budworm (*Heliothis virescens*) (Hilder et al., 1987). Stacking of modified oryzacystatin-I (Oc-IΔD86) and CpTI genes into Arabidopsis plants reduced the number and fecundity of *Heterodera schachtii* females feeding on the transgenic plants (Urwin et al., 1998). This multiple gene approach is considered imperative to reduce the potential for breakdown of conferred plant resistance under pest pressure in the field (Burrows et al., 1998).

Proteolytic activity has long been known to be present in all portions of the pineapple plant (Rowan and Buttle, 1994). Bromelain is a crude, aqueous mixture of enzymes, glycoproteins and carbohydrates extracted from pineapple, the enzymatic fraction of which includes cysteine proteases (EC 3.4.22.31 ananain, EC 3.4.22.32 stem bromelain and EC 3.4.22.33 fruit bromelain) (Maurer, 2001). Inhibitors of these proteases are known to be present in the arial

portions of the pineapple (Yamada et al., 1976; Ota et al., 1972; Lee et al., 1997) as well as the roots (Ham, Paull and Uruu, 1996, unpublished results). Pineapple fruit PIs contain approximately 50 amino acids with a MW of about 5,600 (Reddy, 1975). The role of PIs in nematode population development in pineapple roots is unclear.

Chapter 2

Objectives

Management strategies for control of reniform nematodes in pineapple have focused primarily on chemical measures coupled with a fallow period to reduce initial nematode numbers (Rohrbach and Apt, 1986; Caswell et al., 1990). Recently, interest in alternative methods of control such as the use of nematicidal cover crops has been increasing (Wang and Sipes, 2000). Members of the Cayenne group of pineapple clones are the most widely grown pineapple varieties because of their desirable agronomic characteristics, but are characterized by a susceptibility to nematodes (Purseglove, 1988). The only pineapple clones that show any resistance to reniform nematodes have undesirable agronomic characteristics and are excellent hosts for *P. brachyurus* (Py et al., 1984). Genetic transfer technologies may potentially contribute to the development of pineapple clones resistant to nematode attack (Lilley et al., 1999; Urwin et al., 1998; Urwin et al., 1995).

PI proteins are among the defensive chemicals in plant tissues that are both developmentally regulated and induced in response to insect/pathogen attacks (Ryan 1990). PI proteins form complexes with the proteases of the plant attacker and inhibit their proteolytic activity (Neurath 1984). Since cysteine proteases are thought to be most important for proteolysis in the midgut of nematodes (Koiwa et al., 1997), management of PI levels in plant roots may therefore represent a new method of nematode control. Rice plants were found

to contain a cysteine proteinase inhibitor that reduces the growth of nematodes, and the gene for the inhibitor (OC-I) can be transferred into other plants, such as tomato. Growth retardation of the potato cyst nematode has been successful using this technique (Lilley et al 1996).

Little is known about the concentration of PI in pineapple roots and possible changes in root PI levels over the course of root development. Also, no information is available on the distribution of PI in the roots of pineapple plants. This study was therefore conducted to 1) determine the levels of PI in pineapple roots, 2) determine if there is a relationship between reniform nematode population and PI concentrations in the root over the course of plant development, and 3) determine the concentration of PI along nematode inoculated and not inoculated roots of pineapple plants.

Chapter 3

Materials and Methods

PI and nematodes in roots over time

A Completely Randomized Design experiment with 26 treatments (13 months and +/- nematodes, 4 plants per treatment) was conducted. Pineapple crowns, *Ananas comosus* F153 hybrid, were selected for uniformity of weight (120-150 g) and appearance and planted (3/15/00) into a sand and steam-sterilized soil mixture (1:1) in 25-cm-diameter clay pots. Pots were arranged on two benches in a greenhouse at the University of Hawaii at Manoa campus. One day after planting, half of the plants were inoculated by pipetting 5 ml of water containing 5,000 eggs of *Rotylenchulus reniformis* into a small hole in the soil near the base of the crown. The remaining plants were treated in the same way with 5 ml of nematode-free water. Nematode inoculum was collected from cowpea cultures maintained in the greenhouse (Hussey and Barker, 1973). Plants were watered as needed and every month each plant received 500ml of solution containing 1.2g (half the recommended rate) of 10-20-20 Peters Professional® water soluble fertilizer to provide 0.12g N, 0.24g P_2O_5 and 0.24g K_2O of Peter's 10-20-20 plant food. Starting at 1 month after planting, plants were harvested at monthly intervals for the next 13 months. The experiment was repeated 5.5 months later (8/24/00), with data collected for 8 months.

At each monthly harvest, 4 plants with nematodes and 4 plants without nematodes were randomly selected, photographed, and removed from the pot.

Soil was rinsed from the roots with water, and the length of the longest root of each plant was recorded. All roots were then removed from the plant and shaken in 0.05% NaOCl for 4 minutes to extract nematodes (Hussey and Barker 1973). Eggs were collected on a 20-µm mesh screen, density centrifuged for 4 minutes at 1400 x g, and counted. Roots were saved for inhibitor extraction, and after excess water was absorbed by paper towels, fresh root weight of each plant was taken.

PI and nematodes along the root

A Completely Randomized Design experiment with 2 treatments (+/- nematodes) was conducted. Pineapple crowns, *Ananas comosus* F153 hybrid, were selected for uniformity of weight (120-150 g) and appearance and planted (8/24/00) into a sand and steam-sterilized soil mixture (1:1) in 25-cm-diameter clay pots. Pots were arranged on two benches in a greenhouse at the University of Hawaii at Manoa campus. One day after planting, half of the plants were inoculated by pipetting 5 ml of water containing 5,000 eggs of *Rotylenchulus reniformis* into a small hole in the soil near the base of the crown. The remaining plants were treated in the same way with 5 ml of nematode-free water. Nematode inoculum was collected from cowpea cultures maintained in the greenhouse (Hussey and Barker, 1973). Plants were watered as needed and every month each plant received 500ml of solution containing 1.2g (half the recommended rate) of 10-20-20 Peters Professional® water soluble fertilizer to provide 0.12g N, 0.24g P_2O_5 and 0.24g K_2O of Peter's 10-20-20 plant food. 9

plants (5 inoculated with reniform nematode, 4 not inoculated) were used. The plants were harvested 8 months after planting (4/23/01).

At harvest, plants were removed from the pot and the soil was rinsed from the roots with water. Roots were cut from the base of the crown, separated and grouped according to length, and divided into root base, middle of root, and root tip. The base section was 10 cm from crown attachment, the tip section was 10 cm from the tip of the root, and the middle section was 10 cm or less of the remaining root. The roots of each section were then shaken in 0.05% NaOCl for 4 minutes to extract nematodes (Hussey and Barker 1973). Eggs were collected on a 20-µm mesh screen, density centrifuged for 4 minutes at 1400 x g, and counted. Roots were saved for inhibitor extraction, and after excess water was absorbed by paper towels, fresh root weight of each section was taken.

A Bradford protein assay (Bradford, 1976) was conducted on the extracted supernatant of each section of roots for 4 plants (2 inoculated and 2 not inoculated).

Inhibitor Extraction and Assay

The roots of plants from both experiments were treated as follows for both inhibitor extraction and inhibition assay (Abe et al., 1992). Roots were cut into 1-2 cm pieces with scissors and mixed. A 5-g tissue sample from each plant or each plant section was homogenized in extraction buffer (0.1 M $NaPO_4$ pH 6.0 + 10 mM NaCl), strained through 8 layers of cheesecloth to remove solid pineapple material, and centrifuged for 10 minutes at 10,000 rpm. Plant extract

supernatant was diluted into an 80% acetone solution and allowed to settle overnight at 2°C. After decanting the acetone, the precipitate containing the inhibitors was put into a vacuum chamber for 2 hours to volatilize the remaining acetone. The precipitate was re-suspended in extraction buffer and boiled for 30 minutes to denature proteins and centrifuged for 10 minutes to form a pellet. The supernatant containing the pineapple PI was decanted and the pellet discarded. A 0.2 ml aliquot of supernatant was used for each sample, and the remaining supernatant was stored at -20°C.

A solution containing 0.1 ml assay buffer (0.5 M $NaPO_4$ pH 6.0 + 10mM EDTA), 0.1 ml 50 mM mercaptoethanol, 0.1 ml papain solution (0.025mg/ml), 0.2 ml pineapple inhibitor solution, and 0.1 ml distilled water was incubated for 10 minutes at 30°C. At the start of the reaction, 0.2 ml 1 mM N-benzoyl-DL-arginine-2-napthylamide substrate was added and the solution was incubated for 20 minutes at 30°C. The reaction was stopped by adding 1ml 2% (v/v) HCL in ethanol and 1ml of 0.06% dimethylaminocinnameldehyde in ethanol. Solutions were incubated at 25°C for 30 minutes to allow for color development. Absorbancy of the reaction mixture was measured against a blank (containing only buffer, mercaptoethanol, water, substrate and stop reaction) at 540 nm on a spectrophotometer. Protease inhibition was calculated by subtracting the spectrophotometer readings from a blank with papain added (Bp). Percent inhibition was then calculated by dividing inhibition by Bp and multiplying by 100.

Data Analysis

Data for both experiments were analyzed for variance and treatment differences separated according to a Waller-Duncan k ratio t-test with SAS (Cary, NC). For the PI and nematodes over time experiment, correlation coefficients between nematode numbers (reproductions) and PI concentration were calculated, along with linear regressions of PI concentration and nematode reproduction.

Chapter 4

Results and Discussion

PI and nematodes in roots over time

In the absence of nematode infection, PI levels in the roots increased with plant age, reaching the highest levels by month 6, followed by a slight decrease and subsequent leveling off (Fig. 1). Initial PI levels (month 2) were higher in fall plants than in spring plants, in contrast to the higher PI levels observed in spring plants at month 4. Asano et al. (1999) found the optimum temperature for cysteine PI activity in soybean to be 40° C. The differences observed in Fig. 1 may therefore be explained by greater temperatures experienced by fall and spring plants in month 2 and 4, respectively (Table 1).

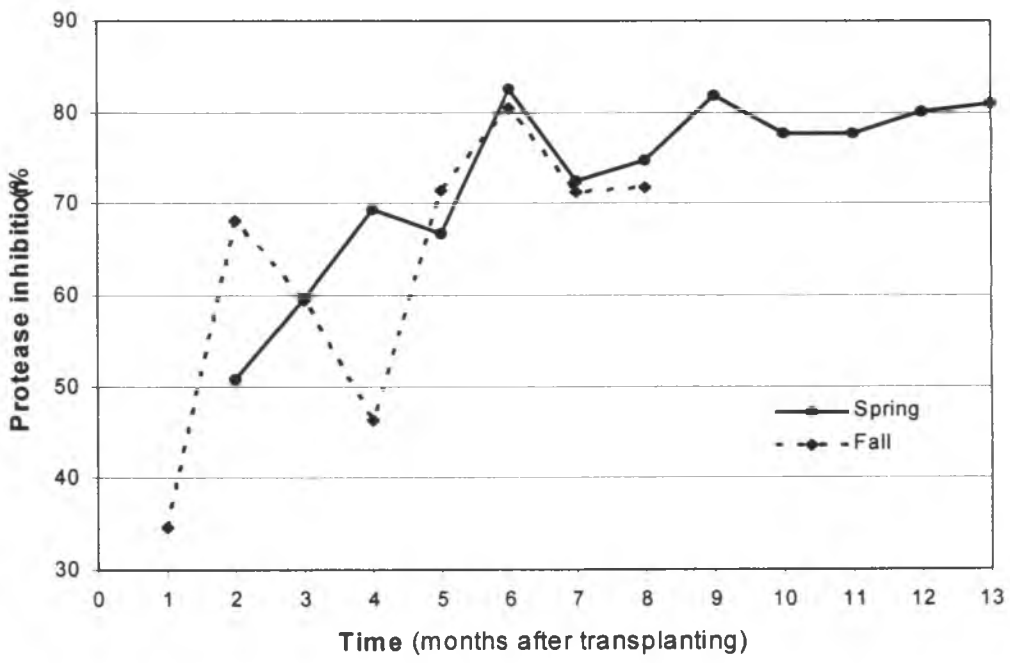

Figure 1. Protease inhibition (%) of papain by extracts of pineapple roots grown in the absence of nematodes. Data points represent mean values of three samples each from four plants.

Table 1. Daily mean temperature measurements from the Honolulu international airport for each month of both experiments. The spring and fall experiments were conducted 3/00-4/01 and 8/00-4/01, respectively. Data courtesy of Hawaii State Climate Office.

Month of experiment	Spring	Fall
1	24	27
2	24	27
3	26	27
4	27	25
5	27	24
6	27	24
7	27	23
8	25	24
9	24	
10	24	
11	23	
12	24	
13	25	

Inoculation with nematodes resulted in higher PI levels in pineapple roots compared to the controls during the first 8 months of plant development, except for month 6 when PI levels of plants not inoculated with nematodes were greater in both experiments (Figs. 2 and 3). In the spring experiment, maximum PI levels were reached after month 8 and were similar for both the inoculated and not inoculated plants (Fig. 2). This early response to nematodes by the plant supports a defensive role for PI in pineapple roots. Root mass and length generally increased over time for both treatments, with no consistent difference between inoculated and not inoculated plants (Table 2 and Fig. 4).

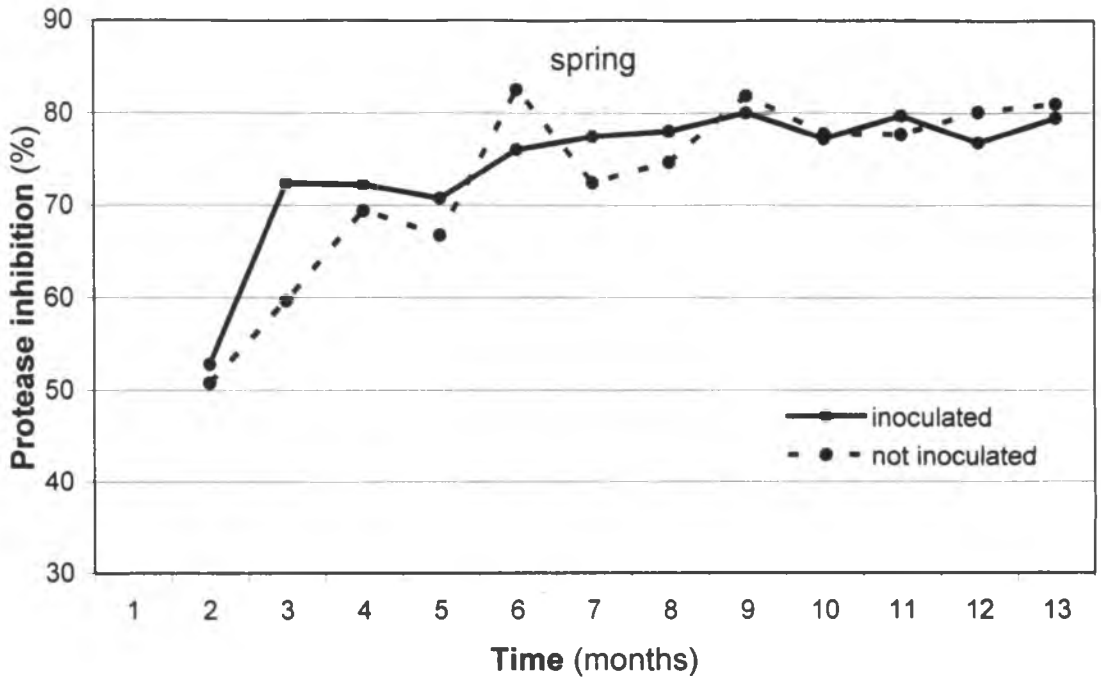

Figure 2. Protease inhibition (%) of papain by extract from pineapple roots inoculated and not inoculated with nematodes in spring experiment. Data points represent mean values of three samples each from four plants.

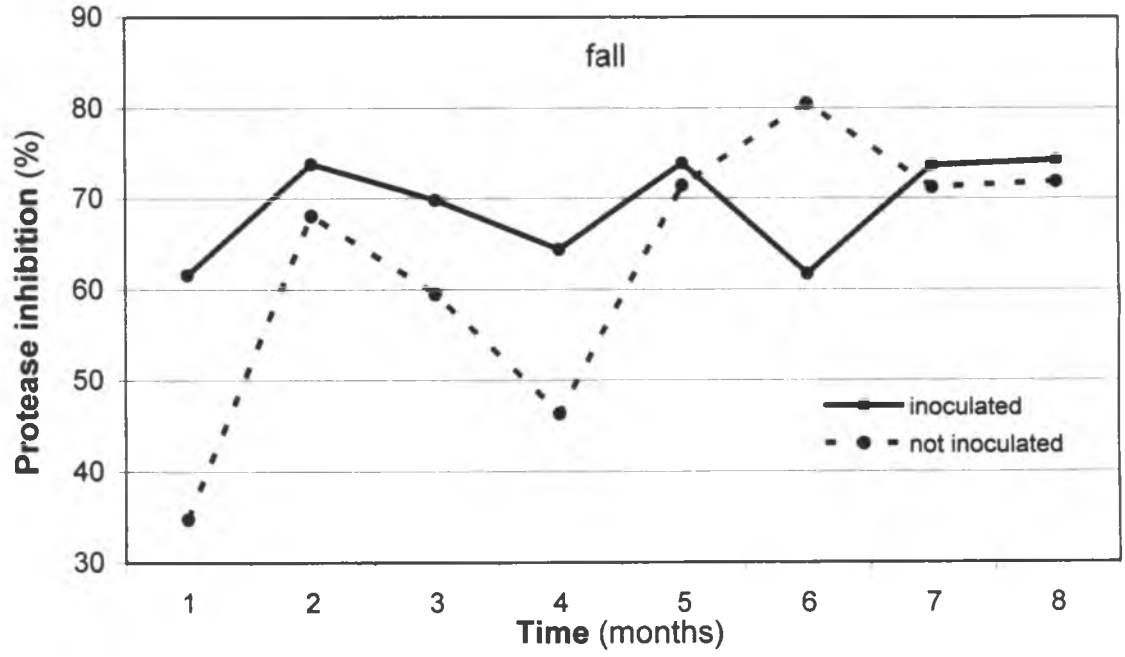

Figure 3. Protease inhibition (%) of papain by extract from pineapple roots inoculated and not inoculated with nematodes in fall experiment. Data points represent mean values of three samples each from four plants.

Table 2. Fresh root weight (g) and length of longest root (cm) in nematode inoculated and control (not inoculated) pineapple plants over time (se = standard error).

Spring	fresh root weight (g)				longest root (cm)			
month	control	se	inoculated	se	control	se	inoculated	se
1	3.55	0.64	3.88	1.13	.	.	.	.
2	11.38	3.45	16.75	1.26	.	.	15.7	0.60
3	13.33	3.85	9.30	2.30	19.2	4.3	15.7	3.28
4	16.35	3.29	15.60	3.46	23	4.3	26.0	4.99
5	12.38	2.30	11.90	1.89	19.6	4.4	22.4	2.60
6	15.98	0.97	14.90	2.50	22	3.5	22.3	2.56
7	14.99	1.39	17.48	1.70	16.5	1.3	17.3	0.48
8	42.54	7.01	36.39	2.41	31.8	5	25.5	1.85
9	22.48	1.75	19.53	3.22	19	2	16	1.47
10	25.75	3.13	38.12	9.34	19.8	2.1	19.8	3.97
11	55.55	10.18	46.07	9.42	31.8	5.6	29.0	2.55
12	41.80	13.72	47.75	17.20	21	2.5	22.5	3.20
13	73.90	12.20	58.10	9.16	38.5	7	29.8	3.47
Fall	fresh root weight (g)				longest root (cm)			
month	control	se	inoculated	se	control	se	inoculated	se
1	0.71	0.31	0.92	0.19	1.95	0.26	2.23	0.36
2	3.89	0.66	2.53	0.65	5.50	0.35	4.75	0.66
3	7.07	2.47	9.89	2.94	7.25	1.60	9.25	1.13
4	18.25	0.96	12.35	1.76	12	1.08	10	0.41
5	19.08	2.55	25.45	2.94	16	2.42	17.00	1.08
6	16.63	1.38	17.73	2.71	15	1.58	18.75	1.11
7	23.90	2.73	29.80	2.78	18	2.86	17.75	1.49
8	23.68	3.81	24.66	1.14	21.25	0.85	21	0.41

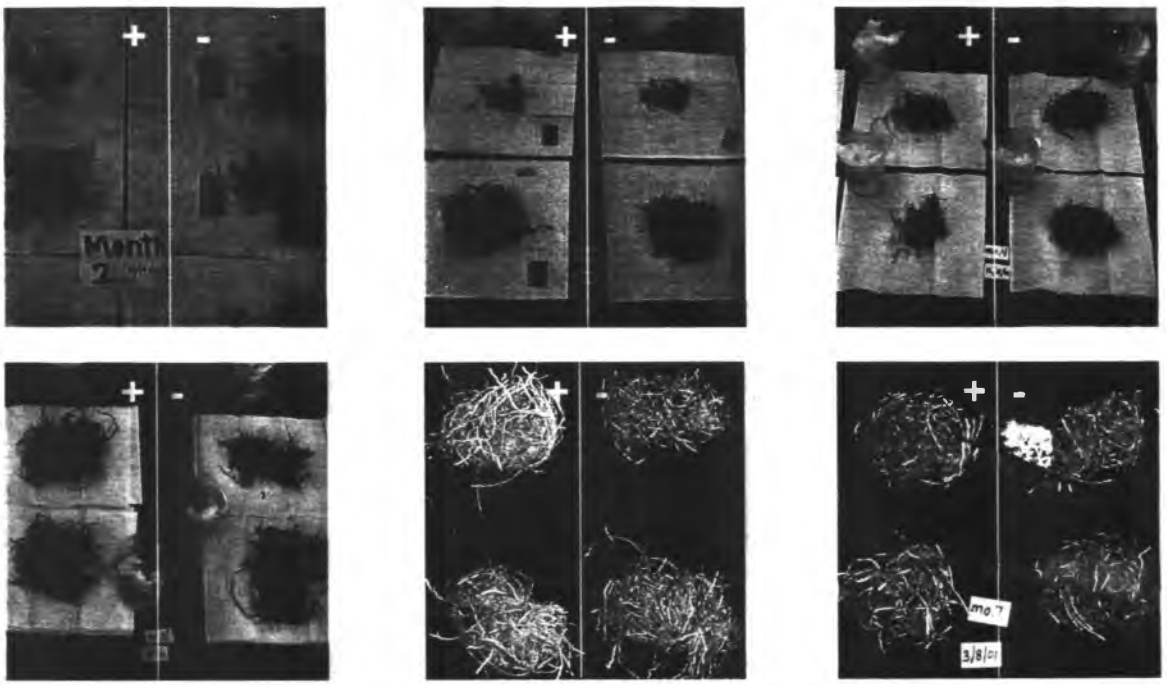

Figure 4. Fall root development from month 2 through 7, with (+) and without (-) nematodes.

Nematode population densities in roots increased to peak levels within the first 6 months of plant growth (Figs. 5 and 6). Peak nematode population numbers occurred 2 months earlier and were 80% greater in spring grown roots (Fig.5) than those from the fall (Fig. 6). This, along with lower fall root mass (Table 2) and poor fall plant development in months 1-3 (Figs. 7 and 8), may reflect sub-optimal (i.e. high) temperature conditions experienced in the fall (Table 1). Optimal daily mean temperature for pineapple root growth is 25°C (Nakasone and Paull, 1998). This temperature was exceeded for the first three months of the fall planting (Table 1). In addition, fall crowns at the time of planting were stressed relative to the spring crowns (i.e., smaller size and brown leaf tips compared to the larger and more lush spring crowns) and may also explain differences in root and nematode growth between experiments.

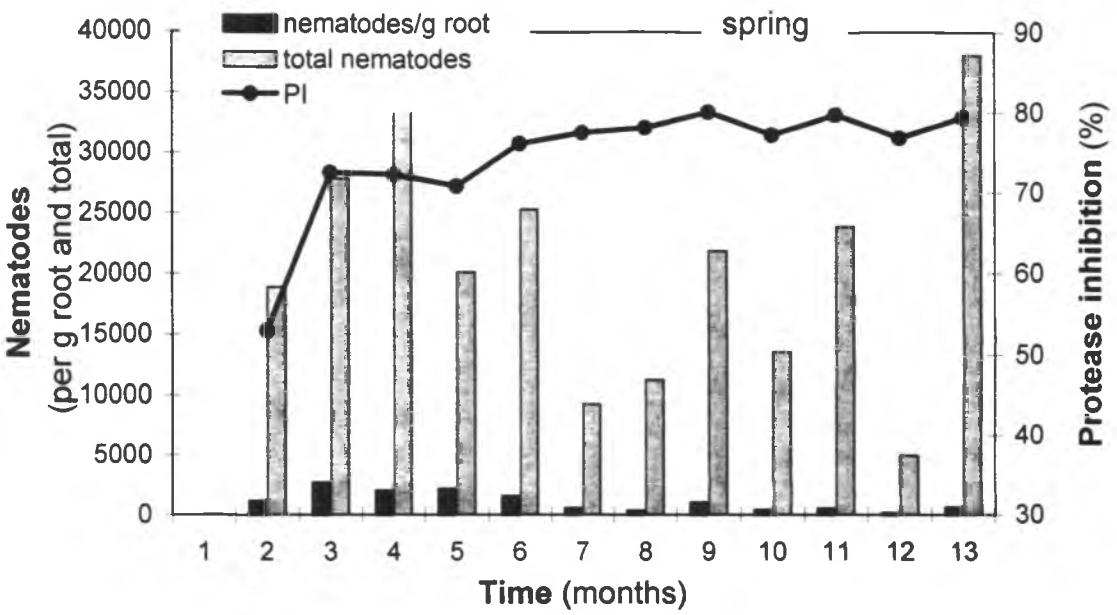

Figure 5. Nematode populations and corresponding protease inhibition of papain (%) by pineapple root extract in spring experiment. PI data points represent mean values from triplicate analysis of four plants. Nematode population data points represent mean values from four plants.

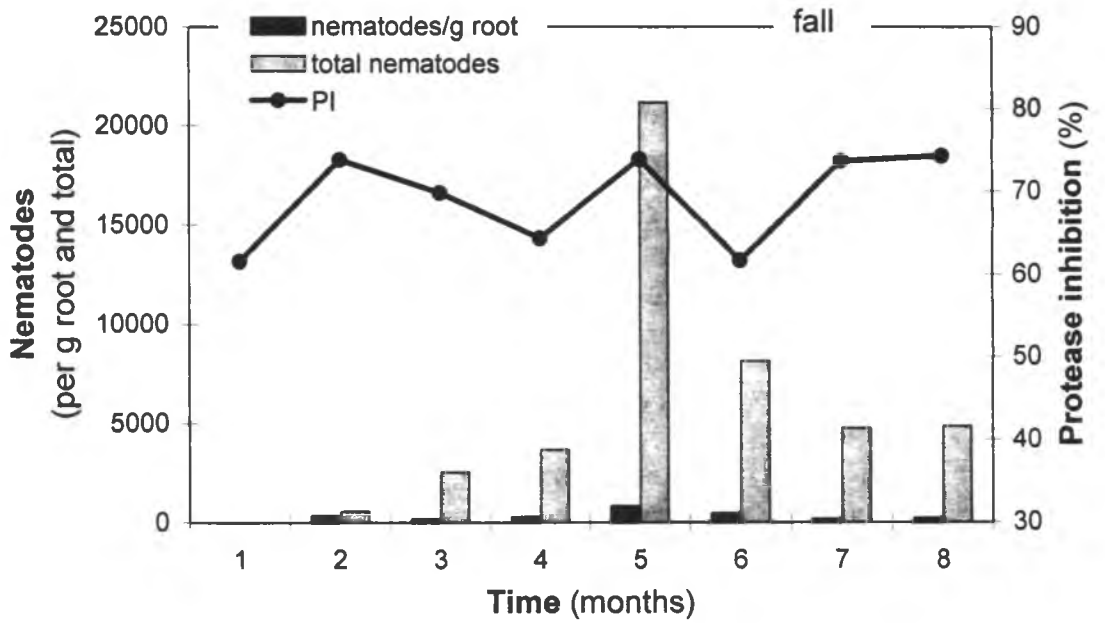

Figure 6. Nematode populations and corresponding protease inhibition of papain (%) by pineapple root extract in fall experiment. PI data points represent mean values from triplicate analysis of four plants. Nematode population data points represent mean values from four plants.

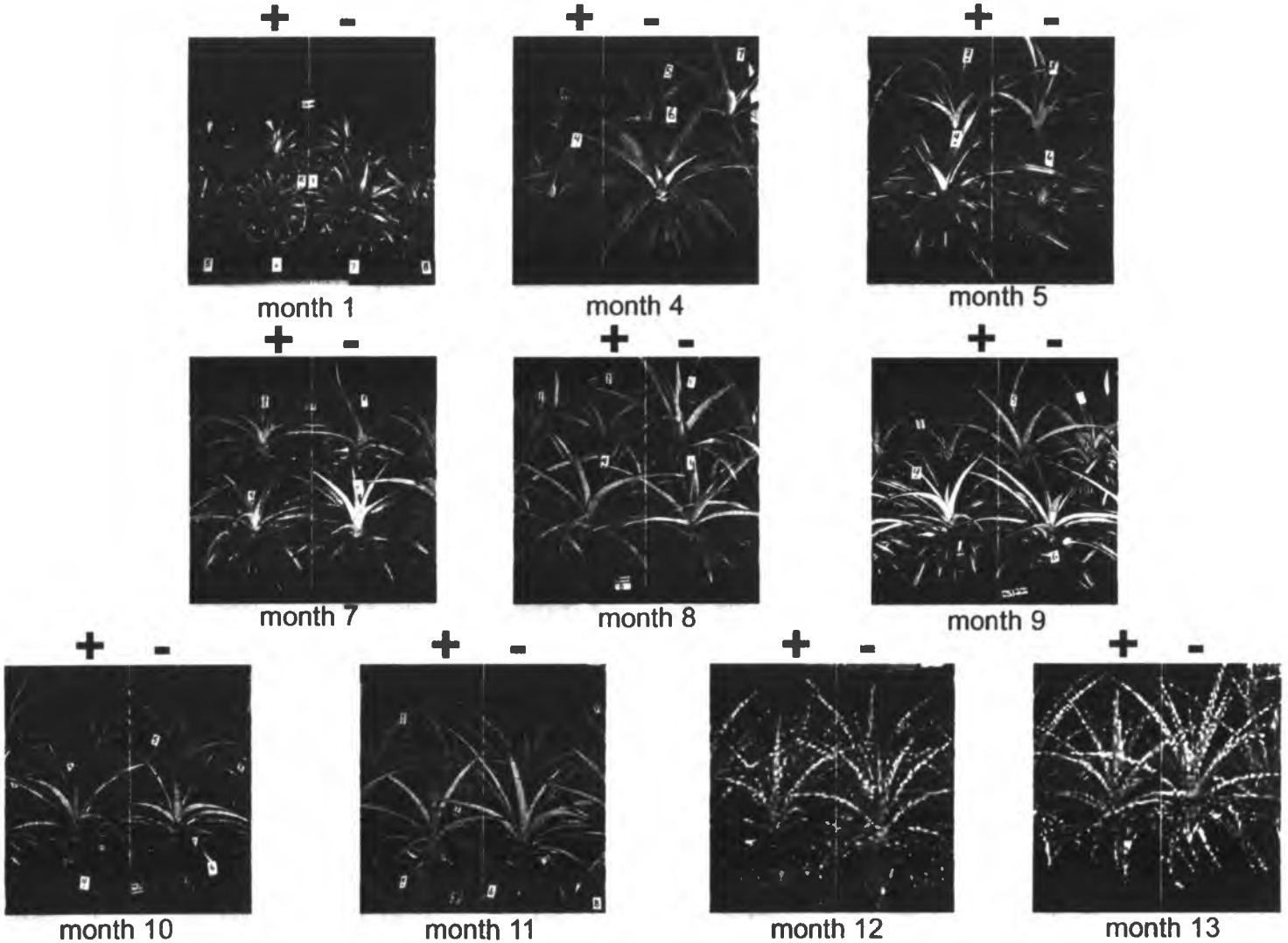

Figure 7. Spring plant development from month 1 through 13, with (+) and without (-) nematodes.

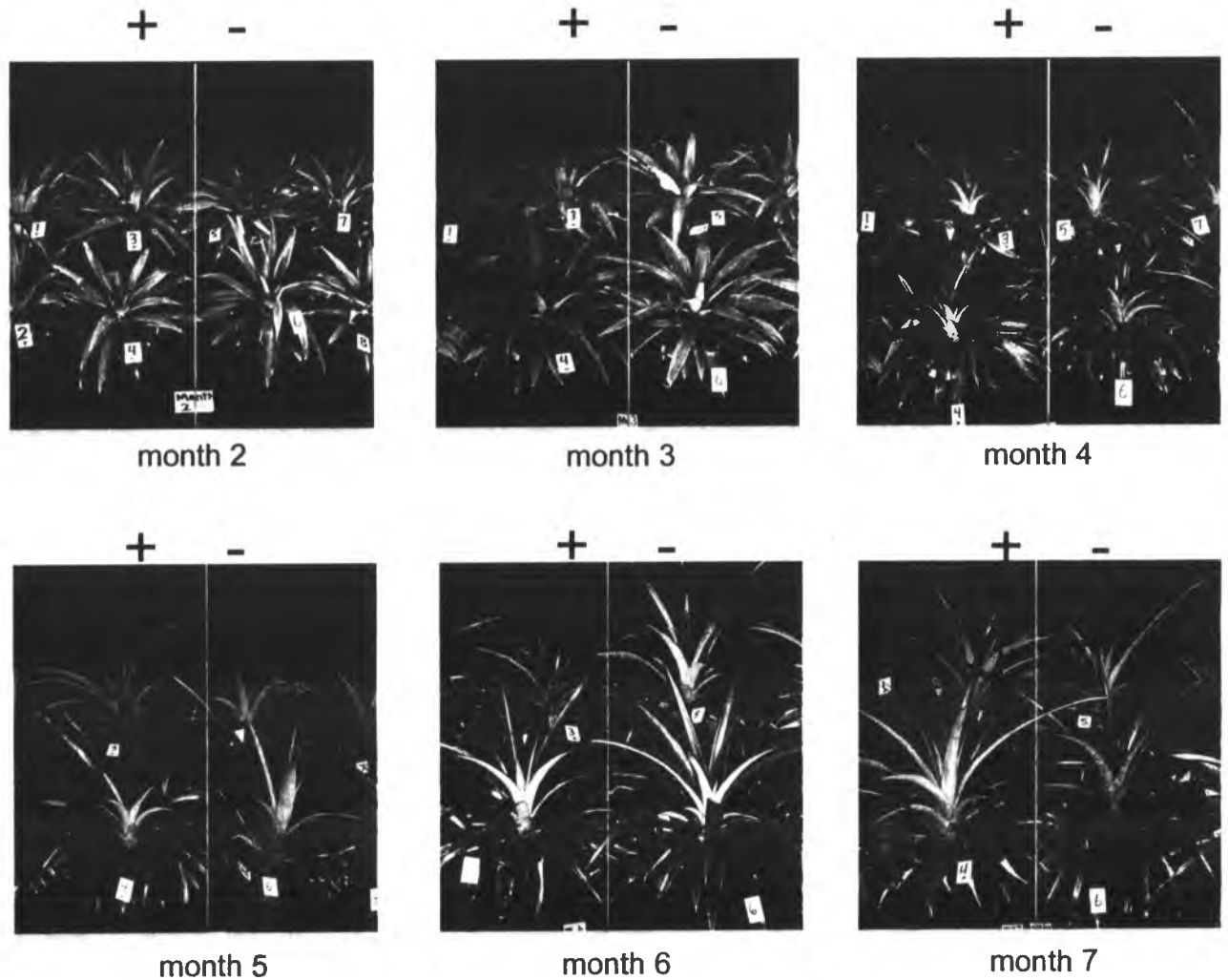

Figure 8. Fall plant development from month 2 through 7, with (+) and without (-) nematodes.

The pattern of nematode population growth reported here differs from reniform nematode population dynamics observed in commercial pineapple fields. Sipes and Schmitt (1994) reported nematode population densities increasing over time, reaching a field carrying capacity within 12 months after planting. After 12 months, the population density remained at approximately field carrying capacity with little or no change. The differences observed in populations between this experiment and those observed by Sipes and Schmitt (1994) are due to one or more reasons. The field nematode counts were taken from soil, not roots as in the counts reported here. Also, field grown plants were not inoculated; natural colonization may occur more slowly than colonization following inoculation. Finally, the root zone environments of pot and field grown plants differed significantly and hence affected population growth. Relatively high temperatures in the pots and competition between nematodes may have caused the population decline observed after month 6.

There was no correlation over the course of plant development between nematode numbers and PI concentrations in the roots, although lower PI concentrations corresponded with greater nematode populations in the early roots of spring grown plants relative to plants grown in the fall (Figs. 5 and 6). The lack of correlation between the two variables possibly reflects either a lag in response time of nematode numbers to PI levels or the absence of a relationship between the two. However, because nematode populations developing in the absence of PI were not observed in this study, conclusive statements on the cause-effect relationship between PI and nematode levels cannot be made.

More work involving treatments of multiple PI concentrations needs to be done to determine if root PI concentrations can affect nematode populations.

PI and nematodes along the root

The concentration of PI was significantly greater ($P<0.04$) in inoculated pineapple roots than in roots not inoculated with nematodes. The difference was observed primarily in the basal portion of the roots (Fig. 9), which corresponded with higher nematode populations in this section of plant roots relative to lower levels in the mid and tip sections (Fig. 10).

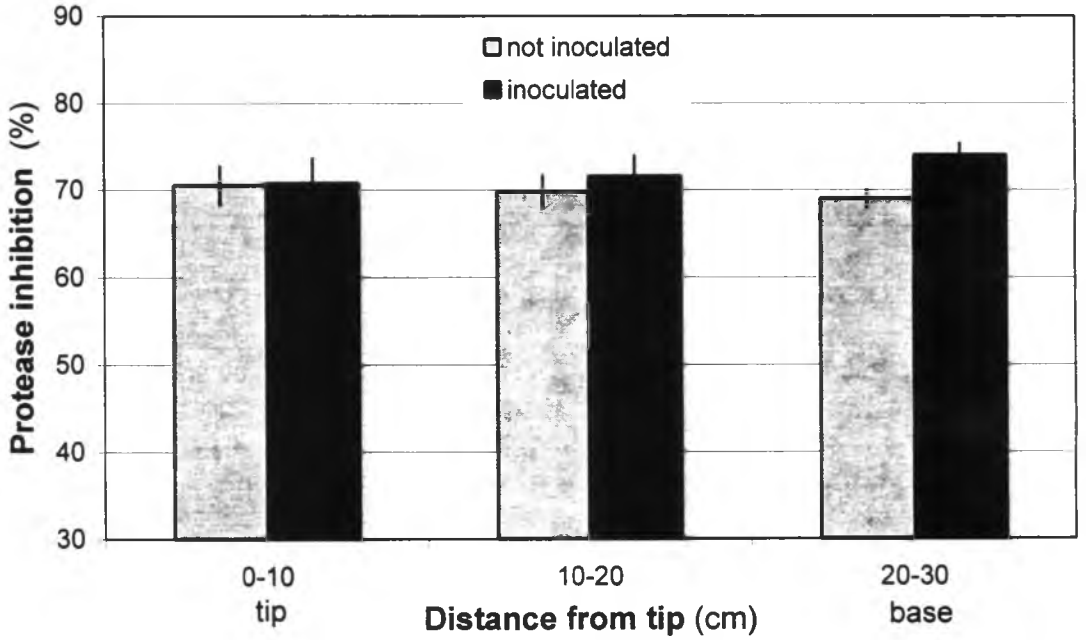

Figure 9. Protease inhibition (%) in different sections of nematode inoculated and not inoculated pineapple roots. Values are means of triplicate analysis of samples from five plants in the inoculated treatment and four plants from the not inoculated treatment. Error bars represent mean error.

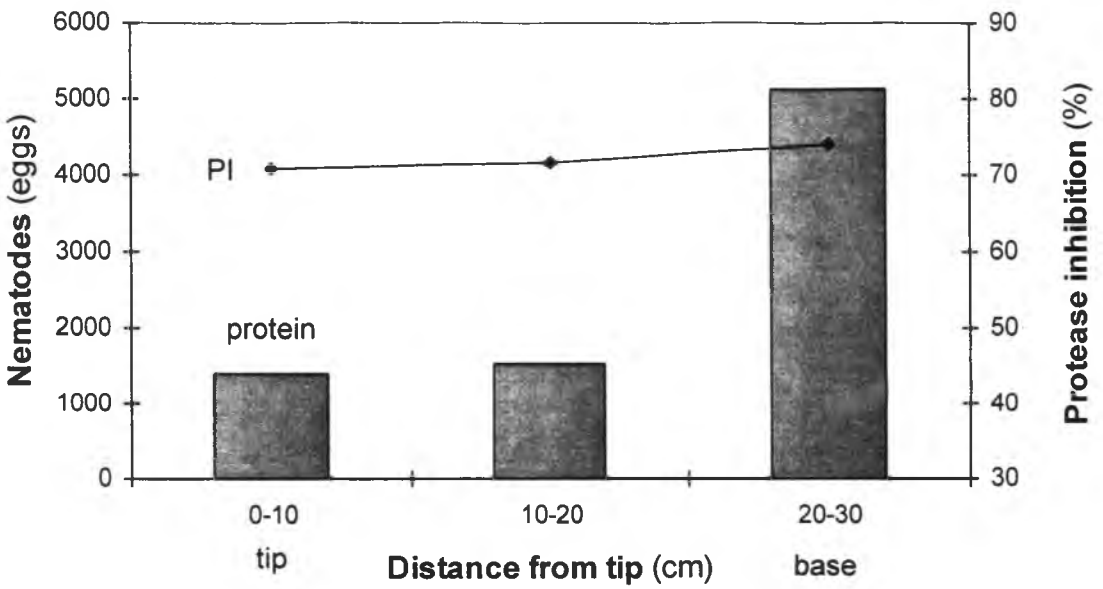

Figure 10. Protease inhibition (%) and nematode densities in different sections of nematode inoculated pineapple roots. Values are means of triplicate analysis of samples from five plants. Error bars represent mean error. Total nematode counts include eggs and vermiform nematodes.

Total root protein decreased with increased distance from the tip in both inoculated and not inoculated treatments (Fig. 11). This followed the pattern of PI distribution in roots not inoculated with nematodes (Fig. 12), but contrasted with the reverse trend of PI distance in inoculated roots (Fig. 13). Lower protein levels in the tip section of inoculated roots (Fig. 11), without a corresponding decrease in PI levels (Fig. 9), and higher protein levels in base and mid sections (Fig. 11) may reflect PI production at, or movement of PI to, the sites of greatest infection.

The greater PI production in inoculated plants and the positive relationship between nematode numbers and PI concentration along the root (Fig. 10)

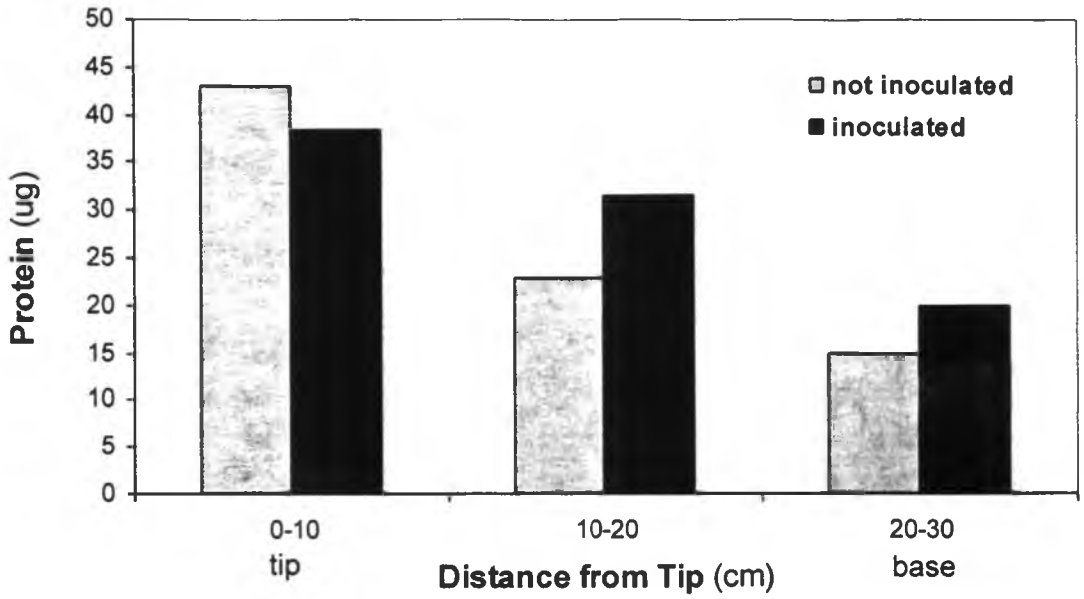

Figure 11. Protein (µg) in different sections of nematode inoculated and not inoculated pineapple roots. Values are means of triplicate analysis of samples from 2 plants in the inoculated treatment and 2 plants in the not inoculated treatments.

supports the role of increased PI production as an induced defense response to nematode attack in pineapple roots. The trend of higher nematode numbers in the base of the root (Fig. 10) is most likely due to the fact that this portion is oldest and has therefore had the most opportunity to be colonized by nematodes. Within treatments, PI concentrations did not differ along the roots (Fig. 9). While PI distribution may have actually been uniform along the roots, trends apparent in Fig. 9 suggest otherwise. Root sections taken for analysis may have been too large and/or too close together along the root to pick up localized differences. Decreasing the section length and increasing the distance between sections may improve the sensitivity of analysis to possible PI gradients along roots.

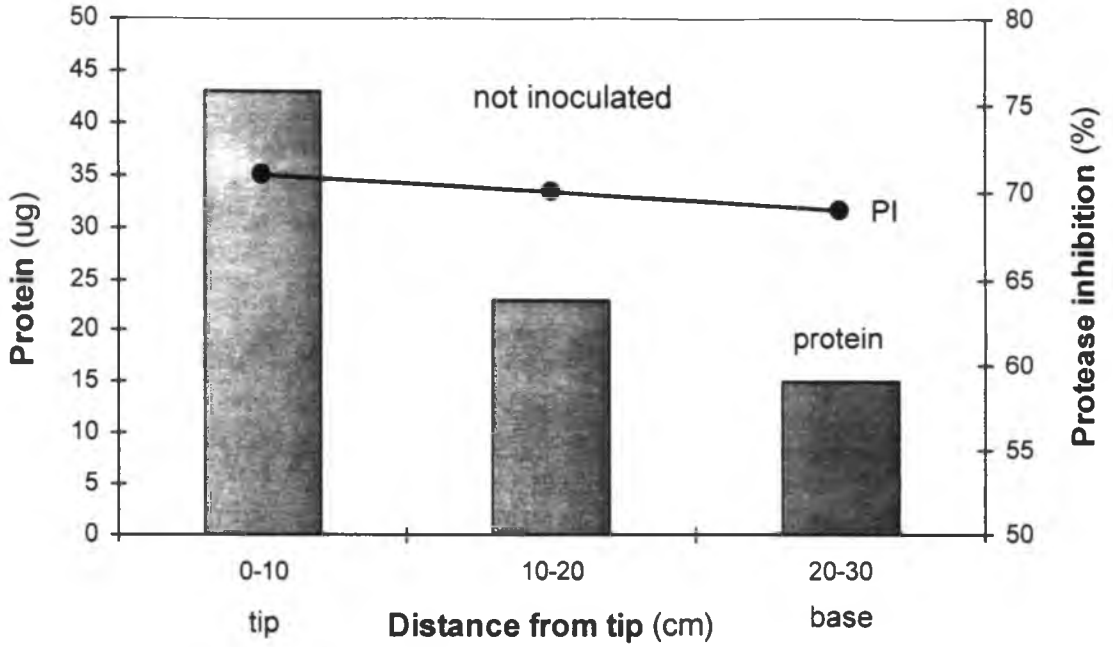

Figure 12. Protein (µg) and Protease inhibition (%) in different sections of pineapple roots not inoculated with nematodes. Values are means of triplicate analysis of samples from 2 plants in the not inoculated treatment.

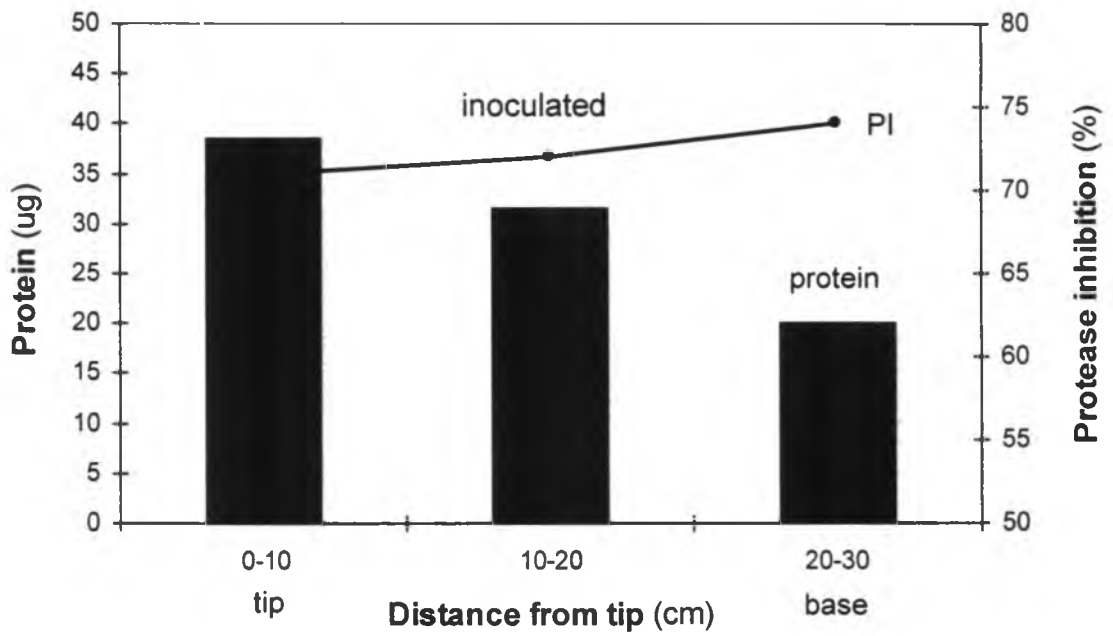

Figure 13. Protein (µg) and Protease inhibition (%) in different sections of nematode inoculated pineapple roots. Values are means of triplicate analysis of samples from 2 plants in the inoculated treatment.

Chapter 5

Conclusions

PI and nematodes in roots over time

PIs are present and increase over time in roots of plants both inoculated and not inoculated with nematodes. During the first 8 months of pineapple plant growth, PI root levels were greater in plants inoculated with nematodes than in plants grown in the absence of nematodes. This suggests that increasing PI levels in roots is a defense response of pineapple to nematode infection. The lack of correlation between the two variables possibly reflects either a lag in response time of nematode numbers to PI levels or the absence of a relationship between the two. Early peaking of nematode populations and their subsequent decline in this study relative to observations in the field were probably due to experimental (i.e., pot-grown) conditions.

PI and nematodes along the root

Pineapple roots produce higher levels of PI in response to nematode attack and this difference was observed in the basal portion of the roots, where nematode densities were greatest in inoculated plants. Within inoculated and not inoculated plants, differences in PI levels along roots were not statistically significant. Within the inoculated treatment, PI levels do not appear related to nematode density along the roots. Further work is needed to determine if

increased PI production induced by nematode infection regulates the pathogen population.

Future Work

Future work should be focused on determining whether increased PI root levels are effective in decreasing nematode infection of pineapple roots, and determining the spatial and temporal distribution of PI in pineapple roots. Population response to PI levels may be characterized by exposing populations to multiple levels of PI, either via exogenous applications of PI extracts, or through the use of clones differing in root PI levels. PI antibodies may be employed in staining root and other tissue sections over time to determine where and when in the plant these compounds are present.

Chapter 6

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